Author’s response to reviews

Title: Lower Vitamin D Levels in Saudi Pregnant Women are Associated with Higher Risk of Developing GDM

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Author’s response to reviews:

Kathryn Hart (Reviewer 3): General:

• Minor grammatical issues throughout - suggest thorough grammar review prior to resubmission, for example ‘of’ used in place of ‘in’ in several places.

Response: Grammar review was done after all comments have been addressed in the revised manuscript.
• Abstract: "among vitamin D deficient and normal women" - replace 'normal' with the relevant specific term - assume you mean Vitamin D 'sufficient'? Since there is controversy about what cut offs are used it is really important to be explicit about the status of your participants.  

Could you also state how many were below 25 or 30nmol/L as these are also accepted cut offs for deficiency which might be more informative than a 50nmol cut off which is likely to characterize most people as deficient.

Response: We thank the reviewer for this comment. We have calculated the prevalence of severe vitamin D deficiency (<25nmol/l) and this was found in 198 subjects (38.4%). We have included this information in the revised results section.

• Background: Pg 5, line 41 - 'prevalence of 26-98% in pregnancy' - can you be explicit whether this range of prevalence is based on the same cut off being applied or represents different cut offs in different studies. If the former are these values all based on a deficiency cut off of 50nmol/L?

Response: We modified the statement accordingly for increased clarity of the cut-off used.

• Methods: Pg 6, line 9 'have been as described before' - remove word 'as'. Appreciate that your methods are described elsewhere but some indication of number approached to achieve 515 and the basis for this sample size would be helpful here. What social or demographic information was collected - will be important when characterising your population, e.g. how was 'Saudi' defined - is this assumed to be an ethnically homogenous group? Again, given the controversy surrounding different methods for Vitamin D analysis I think this should be clearly stated in the paper rather than referring the reader to a previous paper - a clear statement of analytic method is required to allow for swift assessment of the study.

Response: We appreciate the insightful comments for the methods. Statements on how the sample size were determined and additional demographic information were provided in the revised methods including a more detailed explanation of 25(OH)D analysis. To determine sample size, the level of confidence is set at 0.05 and the study power at 80% with the ratio of unexposed to exposed group 1:1 and the frequency of the outcome=10%; the required sample is 438 pregnant women. To adjust for non-response rates, an extra 10% of the calculated sample will be added to sum up with 515 pregnant women.

• Results: Did the approx. 100 women who did not return for OGTT differ in any way from those who did? Based on the recently recognized clinical categories of vitamin D status [20-23], we
classified pregnant women having <50nmol/l as deficient and those having >50nmol/l as non-deficient/normal’ - the references you refer to are original trials and a meta-analysis NOT clinical guidelines. Given the wealth of recommendations (e.g. IOM, EFSA, SACN, Nordic Nutrition) for appropriate cut offs would it be more appropriate to refer to one of these consensus statements as the source of your proposed cut off? Pg 7, line 17 ‘rest 90 (17.5%) were normal’ - rephrase as ‘rest 90 (17.5%) were Vitamin D sufficient’.

Response: Cut-off reference based on local recommendations and consensus statement has been provided. To address the first question we are including in this response the differences between the drop outs and followed up subjects as a table. The table below is not included in the revised manuscript. The statement in question has been rephrased accordingly.

### Anthropometric Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Attended Mean ± SD</th>
<th>Not-Attended Mean ± SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (in Years)</td>
<td>29.1 ± 5.3</td>
<td>28.1 ± 5.6</td>
<td>0.041</td>
</tr>
<tr>
<td>Pre-pregnancy BMI (Kg/m²)</td>
<td>27.0 ± 6.0</td>
<td>27.0 ± 5.9</td>
<td>0.878</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>28.1 ± 6.3</td>
<td>27.8 ± 6.2</td>
<td>0.588</td>
</tr>
<tr>
<td>Waist-Hip Ratio</td>
<td>0.9 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.190</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>113.8 ± 13.2</td>
<td>114.2 ± 11.6</td>
<td>0.737</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>67.4 ± 9.5</td>
<td>68.9 ± 9.8</td>
<td>0.080</td>
</tr>
<tr>
<td>Body Fat %</td>
<td>34.1 ± 5.6</td>
<td>34.8 ± 5.5</td>
<td>0.186</td>
</tr>
</tbody>
</table>

### Biochemical parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>V1 Mean ± SD</th>
<th>V1 Mean ± SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D (nmol/l) at V1 #</td>
<td>27.7 (18.8 - 43.6)</td>
<td>29.7 (21.3 - 44.9)</td>
<td>0.171</td>
</tr>
<tr>
<td>Corrected Calcium (mmol/l) at V1</td>
<td>2.2 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>0.002</td>
</tr>
<tr>
<td>Albumin (g/l) at V1</td>
<td>35.7 ± 4.5</td>
<td>36.8 ± 4.4</td>
<td>0.007</td>
</tr>
<tr>
<td>Piv (mmol/l) at V1</td>
<td>1.1 ± 0.4</td>
<td>1.2 ± 0.4</td>
<td>0.021</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l) at V1</td>
<td>5.2 ± 1.0</td>
<td>5.1 ± 1.1</td>
<td>0.399</td>
</tr>
<tr>
<td>HDL-Cholesterol (mmol/l) at V1</td>
<td>1.3 ± 0.4</td>
<td>1.3 ± 0.3</td>
<td>0.717</td>
</tr>
<tr>
<td>Cholesterol-HDL Ratio at V1</td>
<td>4.0 ± 1.1</td>
<td>4.0 ± 1.0</td>
<td>0.566</td>
</tr>
<tr>
<td>LDL-Cholesterol (mmol/l) at V1</td>
<td>3.2 ± 0.7</td>
<td>3.1 ± 0.8</td>
<td>0.357</td>
</tr>
</tbody>
</table>
Triglycerides (mmol/l) at V1 1.4 ± 0.6 1.4 ± 0.6 0.500

Glucose (mmol/l) at V1 # 4.8 (4.4 - 5.3) 4.8 (4.3 - 5.4) 0.661

Insulin (uU/mL) at V1 # 8.2 (4.6 - 18.3) 8.7 (5.0 - 18.4) 0.861

HbA1c at V1 5.1 ± 0.5 5.1 ± 0.6 0.925

Note: Data presented as Mean and standard deviation for normal variables while Median, 1st and 3rd Quartiles are presented for non-normal variables. # indicates non-normal variables; P-value for Mean differences is obtained from using Independent sample t-test for normal variables and Mann-Whitney U test for non-normal variables.

• Pg 7, line 22 'The pregnant women visiting the clinics for the second time during their 24-28th week of pregnancy, and whose anthropometric and biochemical characteristics were already available, were subjected to OGTT and results analyzed according to IADPSG criteria as given in methods’ - this is not needed in results. Just state return rate/ take up rate for OGTT and how these women differed (if at all) from non-returners (see previous point) and then state the prevalence of GDM.

Response: The statement has been revised accordingly.

• Line 25 '116 (27.7%) out of 419 women were diagnosed as positive for GDM, while others 303 (72.3%) were normal.' - avoid use of word 'normal' as previously recommended. Remove word 'others' - not needed.

Response: The statement has been revised accordingly.

• Line 31 'Various anthropometric and biochemical parameters were determined for the pregnant women using blood samples collected during their first visit, as described in methods. From the OGTT results, the pregnant women were categorized as GDM and non-GDM and two were compared for various anthropometric and clinical parameters (Table 1).’ - again this is not required. Repeats method and results section above.

Response: The statement has been revised accordingly.

• Line 43 'Also, all the obesity indices determined in this study, pre-pregnancy BMI, BMI, waist size, hip size and WHR were significantly higher among the GDM women compared to non-
GDM women.' - give summary p values for this group of results, even if you can only say that they were all <0.05.

Response: The statement has been revised accordingly.

- Your adjustment of odds ratios for potential confounders appears appropriate but were you also able to perform multiple regression analysis to assess the relative contributions of the different variables to GDM risk? Were any of the other variables significantly and uniquely associated with GDM as I assume there is considerable overlap between these as you suggested? What would the best predictive model look like?

Response: Thank you for your comment. To establish the independence of early pregnancy factors with GDM, we performed a multivariable analysis using stepwise Forward logistic regression analysis model. Final model included Pre pregnancy BMI (kg/m2), Hba1c and Vitamin D Deficiency. The results are as follows:

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>95% CI for OR</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre BMI (Kg/m2)</td>
<td>1.06</td>
<td>1.02 - 1.10</td>
<td>0.006</td>
</tr>
<tr>
<td>Hba1c</td>
<td>1.93</td>
<td>1.17 - 3.20</td>
<td>0.010</td>
</tr>
<tr>
<td>Vitamin D Deficiency</td>
<td>3.45</td>
<td>1.55 - 7.68</td>
<td>0.002</td>
</tr>
</tbody>
</table>

- Table 1 - p values cannot be 0 - change to <0.001. As well as looking for statistically significant differences and/ or associations based on GDM status could you also look in more depth at the clinical significance of some of your findings as based on mean values alone these are difficult to ascertain? For example mean TAG levels (whilst significantly different) appear relatively close in absolute terms. Did you investigate the proportion of women who would be classed as having abnormal/ normal TAG and whether this is sig associated with optimal/ sub-optimal Vit D.? Similarly whilst it is statistically significant the correlation between serum Vit D and fasting glucose (Fig 1) is weak and the clinical significance of these findings warrants greater discussion.

Response: P values have been changed to <0.001 as suggested by the reviewer. No significant association was observed between optimal/ sub-optimal Vit D and abnormal/ normal TAG (P = 0.815)
Non Deficient Deficient

Triglycerides (<1.7) 65 (72.2) 301 (71.0)

Triglycerides (≥1.7) 25 (27.8) 123 (29.0)

• Discussion: 'Furthermore, all the obesity indices, pre-pregnancy BMI, BMI, waist size, hip size and WHR were significantly higher in the GDM women compared to non-GDM women suggesting a strong confounder effect from obesity.' - I agree so can this confounding effect be quantified (see previous suggestion re: multiple regression)? This is really important to extend our understanding in this field. The general associations you present have been previously shown (as suggested in your intro) so we now need to know which are the primary drivers (is it obesity, for which Vit D deficiency is co-morbid, or is the Vit D deficiency more important?) in order to inform prevention and intervention.

Response: Confounding effect has already been adjusted in Table 2 where multiple logistic regression analysis is used. In adjustment 2, we have used pre-pregnancy BMI as a confounder. Other obesity indices were excluded to address multicollinearity issues.

• Is there any overlap between the studies covered in the three reviews in refs 23, 24 and 25? If so then this section would benefit from some rewriting to reflect this.

Response: The references had similar findings and we included it to reinforce each other’s findings.

• Line 34 - 'However, a systematic review and meta-analysis to study the link between vitamin D and gestational diabetes (2012) indicated a significant inverse relation between serum 25(OH)D and the incidence of GDM.' - this does not need to start 'however' as the pattern of findings (low Vit D, high risk) is the same as described previously.

Response: The statement has been revised accordingly.

• Line 39 'Also, in a prospective cohort study (Spain, 2015) involving 2382 pregnant women, overall, 31.8% and 19.7% vitamin D insufficiency [25(OH)D3 20-29.99 ng/ml] and deficiency [25(OH)D3 <20 ng/ml], respectively, showed no association between maternal 25(OH)D3 concentration and risk of GDM [26].' - this should start with 'however or conversely' as this DOES report a different direction of effect.
Response: The statement has been revised accordingly.

• Line 54 'This was, despite the increase in circulating 25(OH)D levels that was associated with vitamin D supplementation' - again it would be good to put these results in clinical context - how many of the studies were based exclusively on women who were deficient at baseline? Just increasing vitamin D is unlikely to be enough to exert an effect whereas the effect of moving people from deficiency to sufficiency is more clinically important.

Response: Due to the heterogenous nature of the vitamin D supplementation studies we have restricted ourselves to some of the main findings.

• Conclusion: In line with various previous comments I think that, to advance the field, the conclusion needs to be extended beyond the basic association between Vitamin D and GDM to include some recognition of the potential confounders of this relationship or, ideally, the independence of this association from weight or other potential confounders (if these have been clearly proven and reported in the results).

Response: Thank you for your comment. Relationship between Vitamin D and GDM has also been reported in Table 2 after adjusting for potential confounders. Other changes have been made according to the reviewer’s suggestion.

• Figure 1. Legend can be simplified - first sentence fragment (starting "Correlational analysis of..) is unnecessary as same information is conveyed in fragment starting 'Scatter plot of… Figure legend should include sample size (n= )

Response: It has been changed accordingly.

• Table 1 - title is slightly misleading. Perhaps rephrase to be clear that characteristics are at baseline but GDM status was not determined until visit 2. Cut off used to determine deficiency (with associated reference) should be stated in table footnote.

Response: It has been changed accordingly.

Jennifer Woo (Reviewer 4): Overall, a really important study and could provide important evidence to the literature related to vitamin D deficiency and gestational diabetes risk.
In the Background section, you discuss the extra-skeletal effects of vitamin D, but should focus on the extra-skeletal effects of vitamin D as it relates to pregnancy; Perhaps a brief review of the evidence in regards to vitamin D deficiency and its association with preterm birth, perinatal infections, preeclampsia etc. (Bruce Hollis and Carol Wagner's work).

Response: References as suggested by the reviewer is now included in the background section with focus on the extra-skeletal effects of vitamin D as it relates to pregnancy.

"Despite several studies, the link between gestational diabetes and maternal vitamin D status is still not completely ascertained" - a better word would be conflicting. Perhaps 1-2 sentences related to the studies that have been published where there is and is not an association between vitamin D deficiency and gestational diabetes risk. Again, exploring the conflicts in the literature in regards to vitamin D deficiency and gestational diabetes risk, provides a great backdrop for your study since you have a significant sample size. I know you discuss this in your discussion section, so perhaps a 1-2 sentence summary of the conflicting evidence as it relates to vitamin D deficiency and gestational diabetes risk.

Response: Changes have now been made according to the reviewer’s suggestion.

In regards to your Methods section, were the women supplemented for vitamin D deficiency after the first trimester? ** Important for me as a clinician to know if there was supplementation given to these pregnant women found to be vitamin D deficiency in the first trimester and if so, how much? If you did track the dietary/supplement use of vitamin D, then to add it to Table 1. Did you check vitamin D status again in the 2nd trimester with the blood draw for Oral Glucose Tolerance Test? If you did, then adding to Table 1 the vitamin D levels at visit 1 and then adding a second variable of vitamin D levels at visit 2 should be added.

Response: We thank the reviewer for this comment. As per the Ministry of Health recommendations, all pregnant women in Saudi Arabia are given multivitamins on first prenatal checkup. This multivitamins include vitamin D (400IU). We were more interested as to whether baseline vitamin D deficiency on diagnosis at first visit is a risk factor for GDM. Unfortunately we did not monitor vitamin D supplementation during successive visits but we assumed that those with severe vitamin D deficiency were given supplements to correct their vitamin D status, as standard procedure.
In your results section there were some questions:

1. Why did you adjust for Pittsburgh Sleep Quality Index? There is no rationale given for this, was it significantly different between non gestational diabetic and gestational diabetic groups?

Response: We thank the reviewer for this observation. We removed the PSQI in the list of confounders and indeed it did not affect the results. It has also been removed accordingly in the revised results and tables.

2. Also, since all of the obesity indices were significantly higher in the gestational diabetic group when compared to the non-gestational diabetic group, why did you not control for all of the body mass indices? Since the data would tell you that there is already going to be a bias in the sampling since the gestational diabetic group was more obese and therefore more likely to be vitamin D deficient. It is hard to tell from your data, that the influence of the higher risk of gestational diabetes is due to vitamin D status vs. obesity status of the patient cohort who had gestational diabetes. You do include this in your discussion as a significant con founder, but I wonder if you could reanalyze the data running the logistic regression controlling for the 4 body mass related indices, and to see if vitamin D deficiency truly was associated with gestational diabetes risk. It would strengthen your paper and your argument.

Response: Thank you for your comments. In adjustment 2 (see table 2) pre-pregnancy BMI has already been included in the model to control the confounding effect. However, including other indices for obesity will bring significant multicollinearity issues therefore only 1 measure of obesity is used.

3. Table 1 - Would include dietary or supplement use of vitamin D for both non gestational diabetic group and gestational diabetic groups, and the vitamin D levels at visit #2.

Response: This data is not available but the authors do agree with the reviewer that this is a very important variable that should have been considered.

4. Need to make it clear for the reader that vitamin D status determination was based on visit #2 blood draw or was it based on visit #1 blood draw.

Response: It has been revised accordingly.
5. In your discussion section, you state "Vitamin D deficiency is common in healthy Saudi adults and is more pronounced in females and especially in the younger age groups", yet the next sentence after that is a study that showed vitamin D deficiency in pre and post-menopausal women. It would be great to include a citation showing vitamin D deficiency being prevalent in younger female age groups in Saudi Arabia.

Response: A reference, as suggested by the reviewer, is now included.

6. Conclusion: perhaps adding one sentence in regards to next steps based on your study and the importance of designing a robust randomized control trial to study the impact that adequate vitamin D supplementation has on improving risk of gestational diabetes.

Response: A sentence, as suggested by the reviewer, is now included.